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# Improved amber and opal suppressor tRNAs for incorporation of unnatural amino acids in vivo. Part 1: Minimizing misacylation

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## ABSTRACT

The incorporation of unnatural amino acids site-specifically is a valuable technique for structure–function studies, incorporation of biophysical probes, and determining protein–protein interactions. THG73 is an amber suppressor tRNA used extensively for the incorporation of >100 different residues in over 20 proteins, but under certain conditions THG73 is aminoacylated in vivo by endogenous aminoacyl-tRNA synthetase. Similar aminoacylation is seen with the *Escherichia coli* Asn amber suppressor tRNA, which has also been used to incorporate UAAs in many studies. We now find that the natural amino acid placed on THG73 is Gln. Since the *E. coli* GlnRS recognizes positions in the acceptor stem, we made several acceptor stem mutations in the second to fourth positions on THG73. All mutations reduce aminoacylation in vivo and allow for the selection of highly orthogonal tRNAs. To show the generality of these mutations, we created opal suppressor tRNAs that show less aminoacylation in *Xenopus* oocytes relative to THG73. We have created a library of *Tetrahymena thermophila* Gln amber suppressor tRNAs that will be useful for determining optimal suppressor tRNAs for use in other eukaryotic cells.

**Keywords:** nonsense suppression; frameshift suppression; tRNA; opal suppressor tRNA; unnatural amino acid incorporation

## INTRODUCTION

Incorporation of unnatural amino acids (UAAs) site-specifically in proteins biosynthetically is a powerful tool that is increasingly being used. The primary approach has been stop codon (nonsense) suppression using a designed tRNA with an anticodon recognizing the stop codon. In higher eukaryotes, nonsense suppression by a tRNA chemically aminoacylated with an UAA (tRNA-UAA) is primarily limited to *Xenopus* oocytes, where microinjection of the mutant mRNA and tRNA-UAA is straightforward and electrophysiology allows for sensitive detection of UAA incorporation (Dougherty 2000; Beene et al. 2003). Recently, site-specific UAA incorporation was performed in *Xenopus* oocytes using frameshift suppression of the quadruplet codons CGGG and GGGU, which allowed for the simultaneous incorporation of three UAAs (Rodriguez et al. 2006).

THG73, an amber suppressor tRNA from *Tetrahymena thermophila* with a G73 mutation, has been used extensively to incorporate >100 residues in 20 different proteins (Dougherty 2000; Beene et al. 2003). With THG73 and any other suppressor tRNAs, a key issue is orthogonality. An orthogonal tRNA is one that is not measurably aminoacylated with natural amino acids by endogenous aminoacyl-tRNA synthetases (aaRSs). Unlike its mathematical counterpart, there can be degrees of orthogonality when referring to tRNAs. While the orthogonality of THG73 has been evaluated using in vitro translation in *Escherichia coli* (Clod et al. 1996), wheat germ (England et al. 1999), and rabbit reticulocyte lysate (Rothman et al. 2005), the primary application of THG73 has been for in vivo translation in *Xenopus* oocytes, which is the focus of the present work. The orthogonality of THG73 is acceptable when injecting low quantities of mutant mRNA and/or tRNA-UAA with incubation times <2 d (Saks et al. 1996; Shafer et al. 2004). However, increasing the amount of mutant mRNA and/or tRNA-UAA or incubation times of 2 d—which we will term “excessive” conditions—led to increased aminoacylation of THG73 in vivo with natural amino acid(s) (Rodriguez et al. 2006). This is highly undesirable, because incorporation of natural amino acids at the suppression site leads to a

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heterogeneous mixture of proteins containing an UAA or natural amino acid(s) and limits the quantity of protein that can be produced by UAA incorporation.

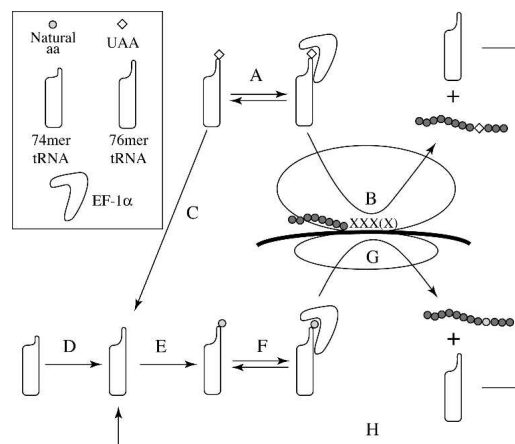
Previous work from our laboratories showed that yeast Phe frameshift suppressor tRNAs (YFFS<sub>CCCG</sub> and YFaFS<sub>ACCC</sub>) were aminoacylated much less than THG73 in vivo, but suppression of the quadruplet codons was less efficient than with THG73 and varied greatly with the amount of frameshift suppressor tRNA injected (Rodriguez et al. 2006). Previously, an *E. coli* Asn amber suppressor (ENAS) tRNA was shown to be highly orthogonal in vivo (Kleina et al. 1990) and in vitro (Cload et al. 1996; Murakami et al. 2003, 2006). Here we show that ENAS has similar amounts of aminoacylation to THG73, suggesting that other well-characterized amber suppressor tRNAs could be prone to aminoacylation under excessive conditions.

Therefore, we chose to determine the amino acid that was being placed on THG73 using the sensitivity of electrophysiology and a well-characterized mutation site of the nicotinic acetylcholine receptor (nAChR), and determined the amino acid to be Gln. Using this knowledge, we created “Knob” mutations on THG73 previously shown to reduce aminoacylation of *E. coli* tRNA<sub>2</sub><sup>Gln</sup> by the *E. coli* glutamyl-tRNA synthetase (GlnRS) (Liu et al. 1997). The Knob mutations on THG73 resulted in tRNAs that showed drastically reduced suppression efficiency in vivo, making them not viable replacements. The *E. coli* GlnRS has been shown to interact with the acceptor stem of the tRNA in the crystal structure (Perona et al. 1989), which was subsequently shown to be sensitive to base pairs and/or backbone positioning of the tRNA acceptor stem (Jahn et al. 1991; Ibba et al. 1996; McClain et al. 1998). We created seven unique tRNAs with mutations in the second to fourth positions of the acceptor stem and analyzed their function in vivo relative to THG73. Mutations in the acceptor stem decreased aminoacylation significantly. This library of *T. thermophila* Gln amber suppressor (TQAS) tRNAs can be used to screen for orthogonality in other eukaryotic cells. To show the generality of the acceptor stem mutations, we also created *T. thermophila* Gln opal suppressor (TQOpS' and TQOpS) tRNAs that are also aminoacylated less than THG73. The acceptor stem mutations have general application for the creation of orthogonal suppressor tRNAs for UAA incorporation in higher eukaryotic cells, where random mutagenesis combined with high-throughput screens are not readily applicable.

## RESULTS

### Schematic for site-specific UAA incorporation and aminoacylation

Figure 1 shows a schematic for the steps leading to UAA incorporation in vivo. Upon injection of the tRNA-dCA-UAA, the tRNA can bind to EF-1 $\alpha$  and/or other



**FIGURE 1.** Site-specific UAA incorporation. (A) tRNA-dCA-UAA binds EF-1 $\alpha$  and/or other components of the translational machinery. (B) UAA is incorporated at the suppression site [XXX(X)], resulting in a protein with an UAA. (C) Undesired hydrolysis and/or aaRS editing results in irreversible loss of the UAA, resulting in tRNA-dCA (76 mer). (D) Injection of the tRNA (lacking dCA, 74 mer) is converted to a 76 mer in vivo by the addition of CA (tRNA-CA). (E) Undesired recognition of the suppressor tRNA-dCA/CA by endogenous aaRS(s) can result in aminoacylation of the tRNA with a natural amino acid. (F) tRNA-dCA/CA-amino acid binds EF-1 $\alpha$  and/or other components of the translational machinery. (G) Undesired protein translation can occur by placing a natural amino acid at the suppressor site (XXX[X]), rather than termination at the stop codon or a frameshift, and this competes with UAA incorporation. (H) After translation, the tRNA-dCA/CA is released into the cytoplasm and can repeat steps E-H.

components of the translational machinery in a reversible fashion (Fig. 1A). It has been shown that the prokaryotic ortholog EF-Tu can protect tRNA-dCA-UAA from hydrolysis in vitro (Fig. 1C; Nakata et al. 2006), thus favoring incorporation of UAA by recognition of the suppressor site on the mRNA (Fig. 1B). If tRNA lacking the terminal CA (74 mer) is injected, it can be converted to the full-length tRNA by the addition of CA in vivo (Fig. 1D). The full-length tRNA, either tRNA-dCA or tRNA-CA (76 mer), may then be aminoacylated with a natural amino acid by an endogenous aaRS (Fig. 1E). The tRNA-dCA-amino acid can then bind EF-1 $\alpha$  and/or other components of the translational machinery (Fig. 1F) and lead to the incorporation of a natural amino acid at the suppression site (Fig. 1G) in competition with the suppression by tRNA-dCA-UAA (Fig. 1B), a highly undesirable outcome. After translation, both UAA and natural amino acid incorporation causes the tRNA-dCA/CA to be released from the ribosome (Fig. 1H), which can further contribute to the undesired natural amino acid incorporation (Fig. 1E-G).

### Experimental scheme for evaluating aminoacylation and suppression by electrophysiology

All experiments were performed on the nAChR, which has been extensively studied by the site-specific incorporation

of UAAs. The muscle-type nAChR is a pentamer composed of  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  subunits in the ratio of 2:1:1:1, respectively. In order to compare experiments from different batches of oocytes, we normalized the average maximal current in response to 1 mM ACh for each suppressor tRNA to the corresponding average maximal current for THG73. Wild-type mRNA expression would be the most desirable normalization, but injection of 20–60 ng of mRNA (as in the aminoacylation experiments) would produce wild-type currents  $>100 \mu\text{A}$ , resulting in current saturation.

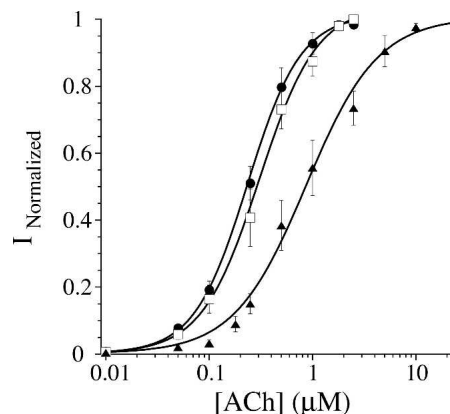
To evaluate aminoacylation, we chose a Leu site in the second transmembrane helix (M2) of the  $\beta$  subunit termed  $\beta 9'$ . This site has been shown to tolerate placement of many natural amino acids and UAAs (Filatov and White 1995; Kearney et al. 1996; Kosolapov et al. 2000), with most causing characteristic shifts in ACh  $EC_{50}$ . Previously, THG73 was shown to incorporate natural amino acid(s) at this site under excessive conditions (Rodriguez et al. 2006). A possible complication for experiments designed to probe aminoacylation is readthrough of the stop codon, which can be tested for by injecting mRNA only into the *Xenopus* oocyte. We find that, at the  $\beta 9'$  site, such readthrough is insubstantial, producing currents that are  $\sim 3.5\%$  of those seen when THG73 is included.

During the course of this research, we noticed variations in aminoacylation depending on whether the oocytes were acquired from *Xenopus* Express or Nasco. *Xenopus laevis* frogs from *Xenopus* Express are caught in Africa, while Nasco frogs are bred in a laboratory and are from a more restricted gene pool (L. Northey, pers. comm.). For each experiment, the figure legends state the supplier(s) of the oocytes.

### Identifying the natural amino acid placed on THG73 using electrophysiology

As noted above, we focus on the  $\beta 9'$  site of the nAChR, which for the wild-type  $\beta 9'L$  has an  $EC_{50}$  of  $50 \mu\text{M}$  for ACh. For the well-characterized  $\beta 9'S$  the  $EC_{50}$  is lowered  $\sim 33$ -fold to  $1.5 \mu\text{M}$  ACh (Kearney et al. 1996), and the lowering of  $EC_{50}$  has been shown to scale with the polarity of the residue introduced at the  $\beta 9'$  position (Kearney et al. 1996).

Injection of  $\beta 9'UAG+THG73$  (74 mer) under excessive conditions produces receptors with an  $EC_{50}$  of  $0.24 \mu\text{M}$  ACh (Fig. 2), which is lower than any substitution previously tested at the  $\beta 9'$  position. When  $\beta 9'UAG+THG73-dCA$  (76 mer) was evaluated, an  $EC_{50}$  of  $0.88 \mu\text{M}$  was obtained. We chose to screen  $\beta 9'Q$  because THG73 evolved to suppress the amber codon with Gln in *T. thermophila* (Hanyu et al. 1986), and many amber suppressor tRNAs are aminoacylated with Gln in vivo (Normanly et al. 1990). The conventional mutant  $\beta 9'Q$  has an  $EC_{50}$  of  $0.31 \mu\text{M}$  ACh (Fig. 2). While the  $EC_{50}$  of  $\beta 9'Q$  and  $\beta 9'UAG+THG73$  (74 mer) are comparable, there is approximately a



**FIGURE 2.** Fits to the Hill equation for  $\beta 9'UAG+THG73$  (74 mer)/dCA (76 mer) and  $\beta 9'Q$ . Filled circles are  $\beta 9'UAG+THG73$  (74 mer) (16 ng per oocyte), open squares are  $\beta 9'Q$ , and filled triangles are  $\beta 9'UAG+THG73-dCA$  (76 mer) (5 ng per oocyte).  $EC_{50}$  values are  $0.24 \pm 0.006$ ,  $0.31 \pm 0.02$ , and  $0.88 \pm 0.08 \mu\text{M}$  ACh, respectively. In each experiment  $n > 3$  oocytes.

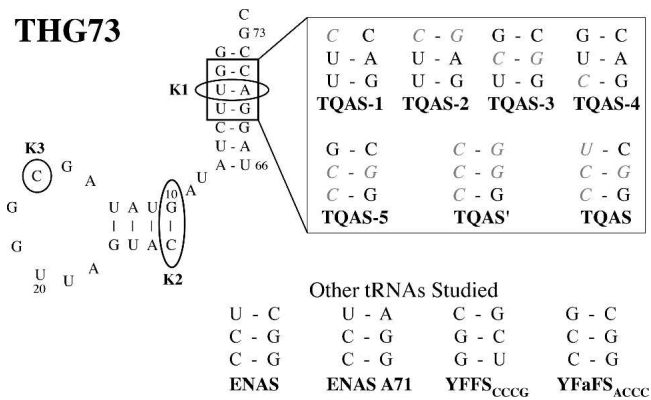
threefold increase in  $EC_{50}$  when  $\beta 9'UAG+THG73-dCA$  (76 mer) is injected (Fig. 2). These results suggest that the full-length THG73-dCA (76 mer) is aminoacylated with amino acids other than Gln. Apparently, this is less important for THG73 (74 mer), which must have CA added in vivo to become a full-length tRNA (Fig. 1D). From these results, we conclude that the predominant natural amino acid placed on THG73 is Gln.

### Testing knob mutations on THG73

Much is known about the recognition of tRNA by the GlnRS, and key interactions have been assigned to various “knobs” on the tRNA, termed K1, K2, and K3 (Fig. 3). In a study of aminoacylation of *E. coli* tRNA<sup>Gln</sup> by the *E. coli* GlnRS (Liu et al. 1997), the mutations that produced the greatest reduction in aminoacylation were K2, K2K3, and K1K2K3. We, therefore, incorporated these mutations into THG73, and tested for orthogonality at the  $\beta 9'$  site.

As shown in Figure 4, THG73 K2, THG73 K2K3, and THG73 K1K2K3 all show  $<20\%$  aminoacylation in vivo when compared to THG73 at  $\beta 9'UAG$  (Fig. 4). However, when each tRNA was chemically ligated with dCA-W and injected with  $\alpha 149UAG$  (a nonpermissive site),  $<3\%$  of the current of THG73-W was seen (Fig. 4). Therefore, THG73 K2, THG73 K2K3, and THG73 K1K2K3 are nonfunctional in the *Xenopus* oocyte and are not viable alternatives for UAA incorporation in vivo.

We conclude that THG73 is very sensitive to the K2 and K3 mutations in the D-stem and D-loop, respectively, which make tertiary contacts with the variable loop to form the characteristic L-shape, tRNA structure. Previous replacement of the THG73 anticodon with ACCC (Shafer et al. 2004) and CCCG (Rodriguez et al. 2006) also resulted



**FIGURE 3.** THG73 mutations and tRNAs studied. Circled positions on THG73 correspond to the knob mutations from Liu et al. (1997), where K1 is C3-G70, K2 is C10-G25, and K3 is G17. The boxed region on THG73 corresponds to the second to fourth positions of the acceptor stem (mutations are shown in gray italics in the right box). Note THG73 K1 and TQAS-3 are the same mutation. Other tRNAs studied are shown at the bottom with only the second to fourth positions of the acceptor stem shown. ENAS and TQAS contain the same nucleotides at these positions.

in nonfunctional frameshift suppressors in vivo. THG73 appears to be exceptionally sensitive to mutation within the D domain and the anticodon loop, which limits the regions where this tRNA can be mutated to create functional tRNAs.

### Testing aminoacylation of ENAS and ENAS A71 in vivo

With the failure of the knob mutations to produce functional tRNAs for UAA incorporation, we chose to screen the ENAS, which was shown to be orthogonal in vitro (Cload et al. 1996) and in vivo (Kleina et al. 1990). When analyzing the structure of ENAS, we noticed that the second position of the acceptor stem contained the non-Watson-Crick base pair U2-C71. We created the variant ENAS C71A to form the canonical pair U2-A71 (Fig. 3) present in the wild-type tRNA (Kleina et al. 1990).

While injection of ENAS or ENAS A71 (74 mer) with  $\beta 9'$ UAG resulted in less aminoacylation than THG73 in both *Xenopus* Express and Nasco oocytes, the effect was much more pronounced with Nasco oocytes (Fig. 5). The  $EC_{50}$ s of ENAS (74 mer) or ENAS A71 (74 mer) with  $\beta 9'$ UAG were  $2.0 \pm 0.1$  and  $1.5 \pm 0.1$   $\mu$ M ACh, respectively, in Nasco oocytes. These  $EC_{50}$ s are larger than the  $0.31$   $\mu$ M ACh for  $\beta 9'$ Q (Fig. 2), suggesting that ENAS and ENAS A71 are aminoacylated by another natural amino acid or a mixture of natural amino acids.

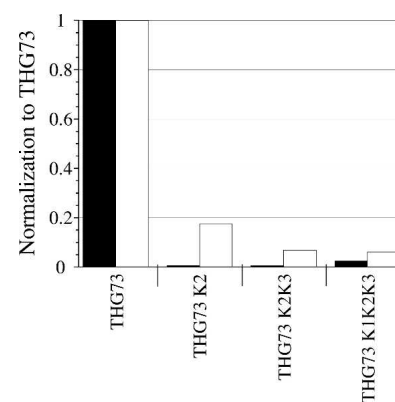
While ENAS and ENAS A71 tRNAs do show improved orthogonality compared to THG73, the suppression efficiencies of ENAS-W and ENAS A71-W were <26% of THG73-W in both *Xenopus* Express and Nasco oocytes (Rodriguez et al. 2007, companion paper, this issue). As

such, neither ENAS tRNA is a viable replacement for THG73 for UAA incorporation in *Xenopus* oocytes.

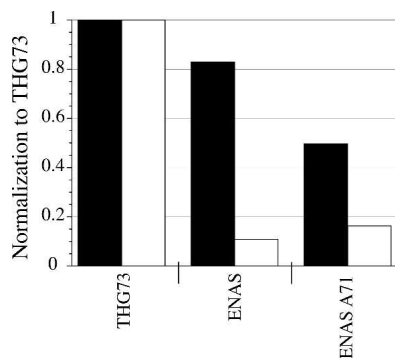
### Testing aminoacylation of THG73 acceptor stem mutations

The *E. coli* GlnRS contacts the tRNA acceptor stem in the crystal structure (Perona et al. 1989), and biochemical experiments have shown specific base pairs and/or positioning of the backbone affect aminoacylation in vivo (Jahn et al. 1991; Ibba et al. 1996; McClain et al. 1998). As such, we decided to screen various mutations in the second to fourth positions of the acceptor stem (shown in Fig. 3) to create a library of *T. thermophila* Gln amber suppressor (TQAS) tRNAs (note in this nomenclature THG73 would be known as TQAS-0). The mutation G2C on THG73 (TQAS-1) results in the second position having C2 C71, which results in a nonfunctional tRNA and serves as a negative control. The single helix pair mutations C2-G71, C3-G70, and C4 are named TQAS-2, TQAS-3, and TQAS-4, respectively (Fig. 3). We then created the double helix pair mutation C3-G70 and C4 (TQAS-5) and the triple helix pair mutation C2-G71, C3-G70, and C4 (TQAS'). Finally, we placed the ENAS acceptor stem from the second to fourth position, including the U2-C71, on THG73 to create TQAS (Fig. 3).

All acceptor stem mutations reduced aminoacylation relative to THG73 in both *Xenopus* Express and Nasco oocytes when tested at  $\beta 9'$ UAG (Fig. 6; Table 1). TQAS-1 shows the same amount of current as  $\beta 9'$ UAG mRNA injection alone, but due to the C2 C71 in the acceptor stem TQAS-1-W is unable to suppress  $\alpha 149$ UAG in both



**FIGURE 4.** THG73 knob mutations. Individual tRNA average current were normalized by appropriate THG73 average current and bars represent this average ratio (total of oocytes tested is 91, where each bar is  $15 > n > 5$  oocytes). Black bars correspond to tRNA-W (21 ng per oocyte) suppressing at  $\alpha 149$ UAG and white bars correspond to tRNA (74 mer) (17 ng per oocyte) +  $\beta 9'$ UAG in *Xenopus* Express oocytes. THG73 knob mutations are <20% when suppressing at  $\alpha 149$ UAG and show they are not functional alternatives for UAA incorporation.



**FIGURE 5.** ENAS and ENAS A71 aminoacylation tested at  $\beta 9'$ UAG. Individual tRNA (74 mer) (17 ng per oocyte) average current was normalized to THG73 (74 mer) average current and bars represent this average ratio (total of oocytes tested is 75, where each bar is  $22 > n > 5$  oocytes). Black and white bars correspond to oocytes from *Xenopus Express* and *Nasco*, respectively. ENAS and ENAS A71 show a large amount of aminoacylation in *Xenopus Express* oocytes after a 2-d incubation, which has not been seen in vitro (Cload et al. 1996) or in vivo (Kleina et al. 1990). Aminoacylation is drastically reduced when tested in *Nasco* oocytes.

*Xenopus Express* and *Nasco* oocytes (Rodriguez et al. 2007, companion paper, this issue). In *Xenopus Express* oocytes, the single helix pair mutations on THG73 all lower aminoacylation, with orthogonality following the order TQAS-2 < TQAS-3 < TQAS-4 (Fig. 6). Reduction is also seen for the single helix pair mutations in *Nasco* oocytes, but the aminoacylation trend is TQAS-4  $\approx$  TQAS-3 > TQAS-2. These results are consistent with previous biochemistry experiments that showed the second and third positions are GlnRS identity elements (Jahn et al. 1991; Ibba et al. 1996), and that removing the wobble (U4-G69) at the fourth position reduces aminoacylation in *E. coli* (McClain et al. 1998).

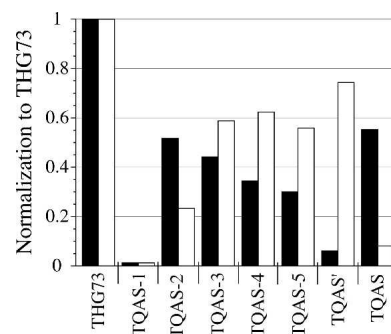
We then combined mutations to reduce aminoacylation further in *Xenopus* oocytes. In *Xenopus Express* oocytes, combining the mutations results in a multiplicative reduction in aminoacylation for TQAS-5 and TQAS' (Table 1), while TQAS, which contains U2-C71, shows the largest amount of aminoacylation (Fig. 6). The overall aminoacylation trend in *Xenopus Express* oocytes is THG73 >> TQAS  $\approx$  TQAS-2 > TQAS-3 > TQAS-4 > TQAS-5 >> TQAS', making TQAS' the most orthogonal tRNA (Fig. 6). The same tRNAs tested in *Nasco* oocytes show no logical trend in combining mutations (Table 1), and TQAS is the most orthogonal tRNA. *Nasco* oocytes display the overall aminoacylation trend THG73 >> TQAS' > TQAS-4  $\approx$  TQAS-3  $\approx$  TQAS-5 > TQAS-2 >> TQAS (Fig. 6). Overall the mutations suggest that in *Xenopus Express* oocytes, C–G pairs in the acceptor stem reduce aminoacylation. *Nasco* oocytes prefer the C–G mutation at the second position (Fig. 3, TQAS-2) or the non-Watson–Crick U–C pair at the second position, along with C–G pairs at the third and fourth positions (Fig. 3, TQAS). While differences in the *Xenopus*

*Express* and *Nasco* oocytes were unanticipated, Figure 6 shows that the TQAS tRNA library contained an orthogonal tRNA for each genetic background.

### Aminoacylation tested at a highly promiscuous site, $\beta A70$

The  $\beta 9'$  site can incorporate many natural amino acids and UAAs (Filatov and White 1995; Kearney et al. 1996; Kosolapov et al. 2000), but we chose to further test aminoacylation of the orthogonal tRNAs selected (TQAS' and TQAS) at another highly permissive nAChR site,  $\beta A70$ . Structural studies place this residue on a highly exposed loop of the receptor (Brejc et al. 2001; Unwin 2005). Previous work on the aligned  $\alpha D70$ UAG showed that it can incorporate the large UAA biocytin with little change in the  $EC_{50}$  (Gallivan et al. 1997). Other work has shown that biocytin is a challenging residue for nonsense suppression (Murakami et al. 2006), supporting the notion that  $\alpha D70$  is a promiscuous site. Therefore, we studied the  $\alpha D70$ -aligned site in the  $\beta$ -subunit,  $\beta A70$ , considering only *Nasco* oocytes.

With the injection of  $\beta 70$ UAG mRNA only, large amounts of current were obtained, indicating that this readthrough represents  $\sim 30\%$  of the current observed in the aminoacylation experiments (Fig. 7). This large amount of readthrough is not typical of most sites studied ( $\beta 9'$ UAG discussed above and  $\alpha 145$ UAG below) and is most likely caused by the high promiscuity for amino acid incorporation. Injection of THG73-dCA and TQAS'-dCA with  $\beta 70$ UAG in *Nasco* oocytes resulted in approximately the same amount of aminoacylation (Fig. 7, white bars), which is consistent with increased TQAS' aminoacylation with *Nasco* oocytes (Fig. 6). However, TQAS shows very little



**FIGURE 6.** THG73 acceptor stem mutations tested at  $\beta 9'$ UAG. Individual tRNA (74 mer) (16 ng per oocyte) average current was normalized to THG73 (74 mer) average current. Bar colors are the same as in Fig. 5. Total of oocytes tested is 189 and each bar is  $26 > n > 8$  oocytes. All mutations in the acceptor stem lower aminoacylation in vivo relative to THG73. TQAS-1 shows lack of aminoacylation but is not accepted by the translational machinery (Rodriguez et al. 2007, companion paper, this issue). TQAS' and TQAS are the most orthogonal tRNAs in *Xenopus Express* and *Nasco* oocytes, respectively.

**TABLE 1.** THG73 acceptor stem mutations

tRNA	Xenopus Express <sup>a</sup>	Nasco <sup>a</sup>	Average <sup>b</sup>
THG73	1.00	1.00	1.00
TQAS-1	0.01	0.01	0.01
TQAS-2	0.52	0.23	0.38
TQAS-3	0.44	0.59	0.52
TQAS-4	0.34	0.62	0.48
TQAS-5	0.30 (0.15) <sup>c</sup>	0.56	0.43
TQAS'	0.06 (0.08) <sup>c</sup>	0.74	0.40
TQAS	0.55	0.08	0.32
U2-C71 (TQAS)	[4.27] <sup>d</sup>	[0.24] <sup>d</sup>	[1.43] <sup>d</sup>

<sup>a</sup>Values from Figure 6.

<sup>b</sup>Average of Xenopus Express and Nasco aminoacylation from Figure 6.

<sup>c</sup>Theoretical values calculated by the multiplication of the single mutations.

<sup>d</sup>Theoretical value for U2-C71 calculated by TQAS/(TQAS-3 × TQAS-5).

aminoacylation compared to THG73 at this site (Fig. 7), and subtracting the mRNA only results in 9% aminoacylation relative to THG73 (also background subtracted).

The EC<sub>50</sub>s for mRNA only, THG73, TQAS'-dCA, and TQAS were 71 ± 1, 56 ± 3, 58 ± 3, and 46 ± 2 μM ACh, respectively, and showed little change from the wild-type EC<sub>50</sub> of 50 μM ACh. These studies further show that TQAS is the most orthogonal tRNA and that TQAS' is aminoacylated extensively in Nasco oocytes.

### LysRS does not aminoacylate THG73, TQAS', or TQAS

THG73 was shown to be aminoacylated by Gln at the β9' site (Fig. 2). However, the 9' position is a conserved Leu in all of the nAChR subunits and forms the hydrophobic gate to the channel (Kearney et al. 1996). Therefore, it is possible that anionic or cationic amino acids such as Asp, Glu, Lys, and Arg may not be incorporated at the β9' position or may produce nonfunctional receptors if they are. This is a concern because many amber suppressor tRNAs have been shown to be aminoacylated by LysRS and/or GlnRS in vivo (Normanly et al. 1990), and mutations to the anticodon stem of an amber suppressor tRNA<sup>Tyr</sup> have shifted the GlnRS or LysRS recognition in vitro (Fukunaga et al. 2006). To evaluate this possibility, we studied a conserved Lys residue in the α subunit of the nAChR, αK145, to test if suppressor tRNAs were aminoacylated by LysRS and/or GlnRS. The conventional mutant αK145Q gave an EC<sub>50</sub> of 144 ± 2 μM ACh (Fig. 8), an approximately threefold increase relative to the wild-type EC<sub>50</sub> of 50 μM ACh.

Injection of α145UAG mRNA alone resulted in very small currents, such that EC<sub>50</sub> could not be determined. Injection of α145UAG with THG73-dCA, TQAS'-dCA, and TQAS resulted in EC<sub>50</sub>s of 167 ± 14, 165 ± 14, and 134 ± 15 μM ACh, respectively (Fig. 8). The EC<sub>50</sub>s for all tRNAs are approximately threefold increased relative to the

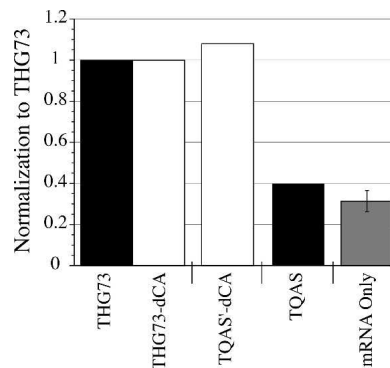
wild-type EC<sub>50</sub>, and therefore all of the tRNAs are aminoacylated by GlnRS and not by LysRS in *Xenopus* oocytes.

### Analyzing THG73-W and TQAS'-W interactions with the translational machinery in vivo

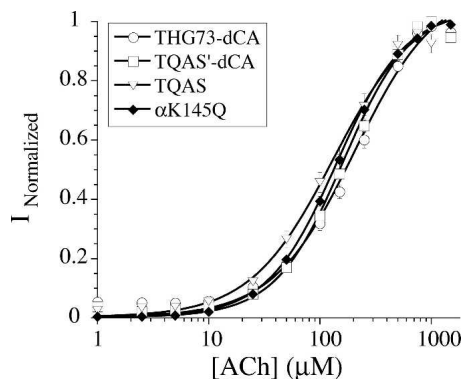
In Figure 1, we outlined the desired events for the incorporation of UAA and the undesired possibility of incorporation of natural amino acids at the suppression site. THG73 was previously shown to be orthogonal to the translational machinery in vitro and in vivo when using less mRNA and/or tRNA along with incubations <2 d (Cload et

al. 1996; Saks et al. 1996; England et al. 1999; Shafer et al. 2004; Rothman et al. 2005). We have shown that with increasing all three of the aforementioned conditions, producing so-called excessive conditions, THG73 can be aminoacylated in vivo. Under these same conditions, TQAS' shows ~90% reduction in the aminoacylation product in *Xenopus* Express oocytes (Fig. 6).

To analyze interactions with the translational machinery we used the temporal control of injection. By injecting tRNA-W first, we analyze the protection of the tRNA-W by EF-1α and/or other components of the translational machinery (Fig. 1A) from the undesired, irreversible loss of the UAA by hydrolysis and/or aaRS editing (Fig. 1C). Subsequent injection of mRNA to assay protein production



**FIGURE 7.** tRNA (74 mer/-dCA) aminoacylation tested at a highly promiscuous site, βA70. All experiments were performed in Nasco oocytes. Black bars are tRNA (74 mer), white bars are tRNA-dCA, and the gray bar is mRNA only. Average currents for TQAS'-dCA and TQAS were normalized to THG73-dCA and THG73, respectively. mRNA only was normalized to THG73. Total of oocytes tested is 60, where each bar is 15 >n>6 oocytes with 9 ng of tRNA per oocyte. mRNA only shows significant readthrough of the UAG stop codon. THG73, THG73-dCA, and TQAS'-dCA all show significant aminoacylation when assayed at β70UAG. TQAS shows comparable currents to the injection of mRNA only, showing less aminoacylation of TQAS in Nasco oocytes.



**FIGURE 8.** tRNA aminoacylation tested at  $\alpha$ K145. Total of oocytes tested is 36, where each dose–response is  $17 > n > 5$  oocytes.  $EC_{50}$  values are  $167 \pm 14$ ,  $165 \pm 14$ ,  $134 \pm 15$ , and  $144 \pm 2 \mu\text{M}$  ACh in the order listed in the legend, with 7.5 ng of tRNA per oocyte. All tRNAs are still aminoacylated by Gln and not Lys (wild-type  $EC_{50} = 50 \mu\text{M}$  ACh).

(Fig. 1B) allows for comparison to what is seen when tRNA-W and mRNA are injected simultaneously. The tRNA–amino acid bond is highly labile at physiological pH. In a recent study, a sample of dCA-Val at pH 7.8 without translational machinery components was 69% hydrolyzed after a 1-h incubation (Wang et al. 2006). The cytoplasm of a *Xenopus* oocyte is estimated to be between pH 7.3 and 7.7 (Webb and Nuccitelli 1981; Sasaki et al. 1992), and therefore some hydrolysis of tRNA-dCA-W would be expected after 3.5 h, if not protected by other proteins. EF-Tu has been shown to protect YFFS<sub>CCCG</sub>-UAA from hydrolysis in vitro, but significant loss was seen after 1 h (Nakata et al. 2006).

Simultaneous injection of THG73-W/TQAS'-W (10 ng per oocyte) and  $\alpha$ 149UAG mRNA gave average maximal currents of  $-3.9 \pm 1$  and  $-4.1 \pm 1 \mu\text{A}$ , respectively, in *Xenopus* Express oocytes. Surprisingly, we see no loss in current from either THG73-W or TQAS'-W when injecting mRNA 3.5 h after the tRNA-W, with  $-3.9 \pm 0.5$  and  $-3.9 \pm 1 \mu\text{A}$ , respectively. Injection of twice as much THG73-W (20 ng per oocyte) resulted in  $-6.3 \pm 2 \mu\text{A}$ . This illustrates that the translation system is not saturated, but there is not a linear increase in current with twice as much tRNA-W in a single injection. Therefore, interactions with EF-1 $\alpha$  and/or other proteins specifically protect both suppressor tRNA-Ws from deacylation by aaRS(s) and hydrolysis, which is not problematic after 3.5 h in the *Xenopus* oocytes.

A double injection protocol, in which a second dose of aminoacyl tRNA is injected 24 h after the first, allows us to assay competition between the suppressor tRNA-W and tRNA-Q (generated by aminoacylation in vivo) after 24 h (Fig. 1B,G). Only aromatic amino acids can function at  $\alpha$ 149UAG, and suppressing with tRNA-W results in wild-type channels. If, however, there is competition with tRNA-Q, then, during the second 24 h, there will be a decrease in the overall protein production or less than double the

current. In the event, a double injection with a 24-h interval of THG73-W/TQAS'-W with  $\alpha$ 149UAG showed currents of  $-8.9 \pm 2$  and  $-8.6 \pm 3 \mu\text{A}$ , respectively, which is greater than twice the protein relative to the single injection. The higher protein production is most likely due to residual mRNA from the first injection, even though nonsense-mediated decay in *Xenopus* oocytes is expected to remove most mRNA with premature stop codons after 18 h (Whitfield et al. 1994).

The double injection studies establish that aminoacylation of the tRNA-dCA in *Xenopus* oocytes (Fig. 1E–H) is not problematic for the first 24 or more hours in vivo for either THG73 or TQAS', because there is no reduction in protein and, therefore, lack of competition for the suppression site. This is surprising because THG73 is aminoacylated  $\sim 90\%$  more than TQAS' in *Xenopus* Express oocytes after 2 d (Fig. 6). This does, however, agree with previous investigations of THG73 orthogonality in vivo with incubations of 24 h or less (Saks et al. 1996).

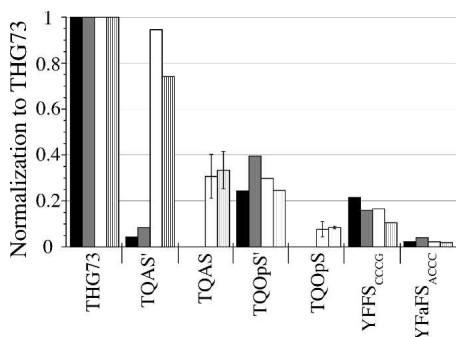
### Comparing aminoacylation of amber, opal, and frameshift suppressor tRNAs

Increasing the number of UAAs to be simultaneously incorporated site-specifically requires the use of a unique stop or quadruplet codon for each UAA. Previously we screened two yeast Phe frameshift suppressor tRNAs, YFFS<sub>CCCG</sub> and YFaFS<sub>ACCC</sub>, in *Xenopus* oocytes and found that both tRNAs were aminoacylated much less than THG73 when tested at the  $\beta 9'$  site. However, their suppression efficiency was decreased relative to THG73 in vivo, although still adequate for UAA incorporation (Rodriguez et al. 2006). Previous work has established that the opal (UGA) and ochre (UAA) stop codons can be suppressed in mammalian cells when using suppressor tRNAs that are aminoacylated by endogenous aaRSs (Capone et al. 1986) or by the import of an exogenous *E. coli* aaRSs (Kohrer et al. 2004). The opal codon has also been used to incorporate an UAA in mammalian cells using a tRNA/synthetase pair (Zhang et al. 2004). Also, an opal suppressor was previously created by replacing the anticodon of THG73 with UCA, but this opal suppressor tRNA (not chemically ligated to an UAA) is aminoacylated in vitro and results in protein translation, or aminoacylation product,  $>90\%$  when compared to wild-type translation. Under the same conditions as the opal suppressor tRNA, THG73 and ENAS showed  $<5\%$  aminoacylation product (Clod et al. 1996), suggesting that the opal suppressor derived from THG73 would be highly aminoacylated in vivo and not viable for nonsense suppression. In *T. thermophila*, the ochre suppressor has been shown to suppress both the ochre and amber codons (Hanyu et al. 1986), and so it would not be viable for the simultaneous incorporation of multiple UAAs.

To test the generality of the acceptor stem mutations at reducing aminoacylation in vivo, we created *T. thermophila*

Gln opal suppressor tRNAs (TQOpS' and TQOpS) by replacing the anticodon of TQAS' and TQAS with UCA.

All four subunits of the nAChR terminated with the opal (UGA) stop codon, and these were all mutated to the ochre (UAA) stop codon to avoid suppression by TQOpS' or TQOpS. The masked construct had an  $EC_{50}$  of  $53 \pm 3 \mu\text{M}$  ACh, comparable to the wild-type  $EC_{50}$  of  $50 \mu\text{M}$ . Injection of  $\beta 9'$ UGA with no tRNA resulted in 2.8% of the current normalized to THG73 (74 mer), versus 1.7% with  $\beta 9'$ UAG. The opal codon thus showed  $\sim 1.6$ -fold greater read-through than the amber codon, which has previously been noted in *E. coli* (Anderson and Schultz 2003). Aminoacylation was tested under identical conditions with THG73, TQAS', TQAS, TQOpS', TQOpS, YFFS<sub>CCCG</sub>, and YFaFS<sub>ACCC</sub> at the  $\beta 9'$  suppression site, stop codon, or quadruplet codon, with tRNA (74 mer) and tRNA-dCA (76 mer). This was done to validate initial screens using only (74 mer) and comparisons between (74 mer) and (76 mer) in Figures 4–7. Comparison of suppressor tRNAs (74 mer) and tRNA-dCAs (76 mer) shows no significant difference for each individual suppressor tRNA in *Xenopus Express* or *Nasco* oocytes (Fig. 9). Once again TQAS' and TQAS showed much less aminoacylation in *Xenopus Express* and *Nasco* oocytes, respectively, relative to THG73. TQOpS' and TQOpS both show less than  $\sim 40\%$  and  $\sim 9\%$  aminoacylation, respectively, compared to THG73 (Fig. 9). This shows that the acceptor stem mutations on THG73 are able to reduce aminoacylation for both amber and opal suppressor tRNAs. YFaFS<sub>ACCC</sub> and YFFS<sub>CCCG</sub> show a similar trend to that previously reported (Rodriguez et al. 2006) with average aminoacylation products of 19% and 3%, re-



**FIGURE 9.** Amber, opal, and frameshift suppressor tRNAs tested at  $\beta 9'$ . All tRNA 74 mer or -dCA (7.5 ng per oocyte) average currents were normalized to THG73 74 mer or -dCA average currents, respectively. Black bars are tRNA 74 mer +  $\beta 9'$  (UAG, UGA, CGGG, or GGGU) and gray bars are tRNA-dCA (76 mer) +  $\beta 9'$  (UAG, UGA, CGGG, or GGGU) in *Xenopus Express* oocytes. White and hatched bars are tRNA 74 mer and 76 mer, respectively, in *Nasco* oocytes. Total of tested oocytes is 276, where  $17 > n > 5$ . TQAS' and TQAS show significantly reduced aminoacylation in *Xenopus Express* and *Nasco* oocytes, respectively, when compared to THG73. Both opal suppressor tRNAs (TQOpS' and TQOpS) show less aminoacylation than THG73. Overall, the frameshift suppressor YFaFS<sub>ACCC</sub> is the most orthogonal tRNA in both *Xenopus Express* and *Nasco* oocytes.

spectively, of THG73. The orthogonality trend is as follows: YFaFS<sub>ACCC</sub> > TQAS' (*Xenopus Express*)  $\approx$  TQOpS > YFFS<sub>CCCG</sub> > TQAS (*Nasco*)  $\approx$  TQOpS' >> THG73, which is aminoacylated the most.

## DISCUSSION

THG73 is an amber suppressor tRNA that has been used extensively for the incorporation of >100 residues in 20 proteins (Dougherty 2000; Beene et al. 2003). Using conditions such as low quantities of mRNA and/or tRNA with incubation times <2 d, THG73 has been shown to be orthogonal in vitro (Cload et al. 1996; England et al. 1999; Rothman et al. 2005) and in vivo (Saks et al. 1996; Shafer et al. 2004). However, increasing the amount of mRNA and tRNA with incubation of 2 d leads to the aminoacylation of THG73 by an endogenous aaRS in vivo (Rodriguez et al. 2006). We show that the *E. coli* Asn amber suppressor (ENAS) and ENAS A71 are both susceptible to aminoacylation by aaRSs of the *Xenopus* oocyte (Fig. 5), even though aminoacylation has not been observed in vitro (Cload et al. 1996; Murakami et al. 2003, 2006) or in vivo (Kleina et al. 1990). This suggests that many well-characterized amber suppressor tRNAs may become aminoacylated in vivo when using increased amounts of mRNA and tRNA along with incubations of 2 d, which is desirable for increased amounts of protein.

By using the sensitivity of electrophysiology and the well-characterized  $\beta 9'$  site, we have been able to show that the natural amino acid placed on THG73 is predominantly, if not exclusively, Gln (Fig. 2). The THG73 (74 mer) and  $\beta 9'$ Q show comparable  $EC_{50}$ s, but THG73-dCA (76 mer) shows an approximately threefold increase in  $EC_{50}$  (Fig. 2). These experiments suggest that THG73-dCA (76 mer) is more readily aminoacylated by other endogenous aaRSs, perhaps due to the unnatural dCA at the 3' end, but the predominant amino acid is still Gln. Similar conditions with the THG73 (74 mer) resulted in equivalent  $EC_{50}$  as  $\beta 9'$ Q, and therefore the active THG73-CA (Fig. 1D) appears to be more stringently recognized by the GlnRS.

Intriguingly, THG73 is very sensitive to the previously discussed knob mutations meant to disrupt recognition by GlnRS, and the derived tRNAs are not viable for UAA incorporation. The K2 mutation alone destroys activity, and combining it with other knob mutations provides little rescue. The K2 mutation resides within the D domain of THG73, which makes tertiary contacts with the variable loop to form the characteristic L-shape tRNA structure. This mutation may result in misfolding of the THG73, but it apparently has little effect on *E. coli* tRNA<sub>2</sub><sup>Gln</sup> (Liu et al. 1997). Interestingly, the K1, or TQAS-3, mutation was shown to be functional by itself on THG73 (Rodriguez et al. 2007), but it is nonfunctional in combination with K2 and K3 (Fig. 4). Replacement of the THG73 anticodon with ACCC (Shafer et al. 2004) or CCCG (Rodriguez et al. 2006)



resulted in nonfunctional frameshift suppressors in vivo. THG73 appears to be sensitive to mutation within the D domain and the anticodon loop but tolerates acceptor stem mutations.

ENAS and ENAS A71 aminoacylation in *Xenopus* Express oocytes is comparable to THG73, but there is a 40% decrease due to the U2-C71 versus U2-A71, respectively (Fig. 5), at the second position of the acceptor stem (Fig. 3). ENAS A71 is more orthogonal, which suggests that the U–A is not recognized by endogenous aaRSs as readily as U–C. The opposite trend is seen in Nasco oocytes, but orthogonality is significantly improved for both ENAS and ENAS A71. Clearly, the identity of the second pair has a strong effect on aminoacylation in vivo.

We hypothesized that similar mutations on THG73 could result in less aminoacylation by the GlnRS, because the identity set includes the second to fourth positions of the acceptor stem (Perona et al. 1989; Jahn et al. 1991; Ibba et al. 1996; McClain et al. 1998). The acceptor stem mutations (shown in Fig. 3) greatly reduced aminoacylation in vivo (Fig. 6; Table 1). In *Xenopus* Express oocytes, the single helix pair mutations could be combined to further reduce aminoacylation in a multiplicative manner (Table 1). In contrast, Nasco oocytes showed a strong dependence on specific sequences, and there was no logical trend based on the single helix pair mutations. This nonclassical identity set has previously been identified on yeast Phe tRNA, where combining mutations could compensate for deleterious single mutations to increase aminoacylation in vitro (Frugier et al. 1998). While variation due to the *Xenopus* Express and Nasco oocytes was unanticipated, the library of six functional TQAS tRNAs was able to find an orthogonal suppressor tRNA for each genetic background. We can anticipate that, as other workers move the UAA methodology into other cell types, different orthogonality issues could arise. The library of tRNAs created here contains diverse mutations in the acceptor stem that should allow for orthogonal tRNA selection in other eukaryotic cells, where high-throughput assays for tRNA screening are currently lacking.

Using the temporal control of injection, we were able to evaluate THG73-W and TQAS'-W interactions with the protein translation machinery and competition with the suppressor tRNAs aminoacylated with Gln after 1 d. Shockingly, there is no change in suppression efficiency when the mRNA and tRNA-W are injected simultaneously or when the mRNA was injected 3.5 h after tRNA-W. These experiments show that both suppressor tRNAs are equally protected by EF-1 $\alpha$  and/or other components of the translational machinery for at least 3.5 h, which has not been observed in vitro with or without EF-Tu (Nakata et al. 2006; Wang et al. 2006). Most importantly, this shows that aaRS editing and/or hydrolysis (Fig. 1C) are not problematic with 10 ng of tRNA-W injected in oocytes. Injection of 20 ng of THG73-W resulted in a 60% increase in current

with a single injection, establishing the lack of saturation at 10 ng, although the response is nonlinear. By injecting mRNA and tRNA-W followed by a second injection of the same after 24 h, we could see if there was competition for the suppression of  $\alpha$ 149UAG by tRNA-Q (Fig. 1B,G). The double injection for both THG73-W/TQAS'-W showed a slightly greater than twofold increase in current, indicating a lack of competition with tRNA-Q slightly after 24 h. This agrees with previous experiments using <24-h incubation times (Saks et al. 1996; Shafer et al. 2004). Aminoacylation of THG73, therefore, takes place after 24 h and is not different from TQAS' in *Xenopus* Express oocytes in the first 24 h. However, TQAS' is aminoacylated  $\sim$ 90% less than THG73 after 2 d. Overall, both THG73 and TQAS' interact with the protein translational machinery similarly in *Xenopus* Express oocytes.

In order to simultaneously incorporate multiple UAAs, it is necessary to use a unique stop or quadruplet codon for each UAA. Previously, three UAAs could be simultaneously incorporated using amber and frameshift suppression with the UAG, CGGG, and GGGU suppression sites (Rodriguez et al. 2006). A second nonsense site, either opal (UGA) or ochre (UAA), would be valuable for the incorporation of four UAAs. However, the *T. thermophila* ochre suppressor recognizes both the ochre (UAA) and amber (UAG) stop codons (Hanyu et al. 1986). The opal codon has been used to incorporate an UAA in mammalian cells using a tRNA/synthetase pair (Zhang et al. 2004) and should be feasible for UAA incorporation in *Xenopus* oocytes. Previous work with an opal suppressor created from THG73 by the replacement of the anticodon with UCA resulted in a large amount of aminoacylation in vitro and production of >90% protein relative to wild-type protein (Cload et al. 1996). These results suggest that the THG73-derived opal suppressor would be aminoacylated much more than the THG73 amber suppressor and would not be able to be evaluated under the conditions tested in this paper. To test the generality of the acceptor stem mutations at reducing aminoacylation, we replaced the anticodon of TQAS' and TQAS with UCA to create TQOpS' and TQOpS, respectively. Both TQOpS' and TQOpS show a great reduction in aminoacylation in vivo relative to THG73 (Fig. 9) and are viable suppressor tRNAs for UAA incorporation at the opal codon (Rodriguez et al. 2007, companion paper, this issue). Intriguingly, there is no significant difference in aminoacylation of TQOpS' in *Xenopus* Express and Nasco oocytes (Fig. 9), which suggests the opal suppressor is recognized by a different aaRS(s) and variation in aminoacylation is caused by the GlnRS, which we have shown recognizes THG73, TQAS', and TQAS (Fig. 8), and the tRNAs show different amounts of aminoacylation depending on the genetic background (Figs. 6, 9).

We have shown that acceptor stem mutations have wide applicability for the creation of orthogonal suppressor tRNAs for the incorporation of UAAs in higher eukaryotic

cells. TQAS' and TQAS still retain slight aminoacylation by the GlnRS in *Xenopus* Express and Nasco oocytes, respectively. Mutations in the anticodon stem have been shown to decrease GlnRS recognition and may further reduce aminoacylation, but these mutations can also lead to increased LysRS recognition (Fukunaga et al. 2006). The acceptor stem mutations have no adverse effect on the suppression efficiency and can increase the suppression efficiency by as much as 40% (Rodriguez et al. 2007, companion paper, this issue). While aminoacylation was shown to be dependent on the genetic background of the oocytes, there is no effect on the suppression efficiencies of the TQAS tRNAs (Rodriguez et al. 2007, companion paper, this issue).

In summary, we have created a TQAS tRNA library that will aid in the identification of orthogonal tRNAs for the use of higher eukaryotic cells. When combined with the evaluation of suppression efficiencies described in Rodriguez et al. (2007), these tRNAs should significantly expand the applicability of site-specific incorporation of UAAs by chemically aminoacylated tRNAs.

## MATERIALS AND METHODS

### Materials

All oligonucleotides were synthesized by Integrated DNA Technologies. NotI was from Roche Applied Science. BamHI, EcoRI, FokI, T4 DNA ligase, and T4 RNA ligase were from NEB. Kinase Max, T7 MEGashortscript, and T7 mMessage mMachine kits were from Ambion. ACh chloride and yeast inorganic pyrophosphatase were purchased from Sigma-Aldrich. dCA and 6-nitroveratryloxycarbonyl protected dCA-W was prepared as reported in Robertson et al. (1989) and Zhong et al. (1998).

### tRNA gene preparation and tRNA transcription

THG73, YFFS<sub>CCCG</sub>, and YFaFS<sub>ACCC</sub> subcloned in the pUC19 vector were previously made (Saks et al. 1996; Rodriguez et al. 2006). Genes for ENAS (sequence from Cload et al. 1996) with flanking EcoRI and BamHI overhangs were phosphorylated using the Kinase Max kit, annealed, and ligated with T4 DNA ligase into EcoRI and BamHI linearized pUC19 vector as described (Nowak et al. 1998). ENAS A71 (original sequence from Kleina et al. 1990 with G1-C72 insertion for T7 polymerase transcription) was created by QuikChange mutagenesis on ENAS in pUC19. Knob mutations from Liu et al. (1997) (K2 is C10-G25; K2K3 is C10-G25 and G17; and K1K2K3 is C3-G70, C10-G25, and G17) on THG73 were created by QuikChange mutagenesis. Acceptor stem mutations on THG73 were created by QuikChange mutagenesis and shown in Figure 3. Replacing the anticodon of TQAS' and TQAS from CUA to UCA by QuikChange mutagenesis created TQOpS' and TQOpS, respectively. All mutations were verified by DNA sequencing (California Institute of Technology Sequencing/Structure Analysis Facility). Template DNA for tRNA lacking the 3'CA was prepared by FokI digestion, and tRNA was transcribed using the T7 MEGashortscript kit with 0.5  $\mu$ L of yeast inorganic pyrophosphatase (40 U/mL in 75 mM Tris, 10 mM MgCl<sub>2</sub>, and pH 7.0). tRNA was desalted using CHROMA SPIN-30 DEPC-

H<sub>2</sub>O columns (BD Biosciences), and the concentration was determined by absorption at 260 nm.

### nAChR gene preparation and mRNA transcription

The masked  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  subunits of the nAChR subcloned in the pAMV vector were previously prepared (Rodriguez et al. 2006). All four subunits terminate with the opal (UGA) stop codon and each UGA was mutated to the ochre (UAA) stop codon to avoid possible suppression by TQOpS' and TQOpS.  $\alpha$ 149UAG and  $\beta$ 9'(UAG, CGGG, and GGGU) were previously prepared on the masked constructs (Rodriguez et al. 2006).  $\beta$ 70UAG,  $\alpha$ 145Q,  $\alpha$ 145UAG, and  $\beta$ 9'UGA were prepared by QuikChange mutagenesis. Mutations were verified by DNA sequencing (California Institute of Technology Sequencing/Structure Analysis Facility). DNA was linearized with NotI, and mRNA was prepared with the T7 mMessage mMachine kit with 0.5  $\mu$ L of yeast inorganic pyrophosphatase. mRNA was purified by using the RNeasy Mini kit (Qiagen) and quantified by absorption at 260 nm.

### dCA and dCA-W ligation to suppressor tRNAs

Seventy-five micromolars (instead of 300  $\mu$ M, because there was no change in ligation efficiency) of dCA or 6-nitroveratryloxycarbonyl protected dCA-W were coupled to suppressor tRNAs by using T4 RNA ligase for 30 min as previously reported (Nowak et al. 1998; Petersson et al. 2002), desalted using CHROMA SPIN-30 DEPC-H<sub>2</sub>O columns, and quantified by absorption at 260 nm. tRNA ligation efficiency was qualitatively determined by MALDI mass spectrometry (Petersson et al. 2002), and all tRNA ligations were identical within each prepared group and >80%.

### In vivo aminoacylation and suppression experiments

Prior to in vivo aminoacylation and suppression experiments, all tRNAs and mRNAs were simultaneously made and normalized by UV and densitometric analysis using AlphaEaseFC Stand Alone (Alpha Innotech). Stage VI oocytes of *Xenopus laevis* were prepared as described (Quick and Lester 1994). All tRNAs were refolded at 65°C for 2 min and 6-nitroveratryloxycarbonyl protected dCA-W was deprotected for 5 min by UV irradiation before injection (Saks et al. 1996). Oocytes were injected with 50 nL of mRNA alone or with tRNA and incubated at 18°C for 44–52 h. For EC<sub>50</sub> determination of  $\beta$ 9'Q (Fig. 2), 3 ng of mRNA in the ratio of 2:1:1:1 for  $\alpha$ : $\beta$ 9'Q: $\gamma$ : $\delta$  was injected; 20 ng of mRNA in a subunit ratio of 2:5:1:1 for  $\alpha$ : $\beta$ 9'UAG: $\gamma$ : $\delta$  or 10:1:1:1 for  $\alpha$ 149UAG: $\beta$ : $\gamma$ : $\delta$  was injected (Figs. 2, 4, 5). Figures 6–8 used the same ratio, but 40 ng to 60 ng of mRNA was injected per oocyte. In Figure 9, 40 ng of mRNA in a subunit ratio of 2:5:1:1 for  $\alpha$ : $\beta$ 9'(UAG, UGA, CGGG, or GGGU): $\gamma$ : $\delta$ . Amounts of tRNA injected are listed with each figure.

### Electrophysiology

Recordings employed a two-electrode voltage clamp on the OpusXpress 6000A (Molecular Devices). ACh was stored at –20°C as a 1 M stock, diluted in Ca<sup>2+</sup>-free ND96, and delivered to oocytes by a computer-controlled perfusion system. For all experiments, the holding potential was –60 mV. Dose–response data were obtained from at least eight ACh concentrations and all

tRNA aminoacylation or suppression comparisons were tested with a single 1 mM ACh dose. Dose–response relations were fit to the Hill equation to determine the EC<sub>50</sub> and Hill coefficient ( $n_H$ ). All reported values are represented as a mean ± SE of the tested oocytes (number of oocytes [ $n$ ] is listed with each figure).

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